ABSTRACT

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MODULATION OF CARDIAC PHYSIOLOGY IN ADULT RAT CARDIOMYOCYTES BY PROTEIN KINASE C AND PROTEIN KINASE A

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This dissertation investigated the biochemical modifications and physiological changes associated with the phosphorylation of adult rat contractile proteins (TnI and TnT) using protein kinase C (PKC) isozymes (α, δ, ε, and ζ) and protein kinase A (PKA). It was hypothesized that the phosphorylation of adult rat cardiomyocytes by various PKC isozymes and PKA have specific substrates on myofibrils. It was also hypothesized that the phosphorylation of adult rat cardiomyocytes by PKC and PKA has alternating physiological effects on Mg^{2+} ATPase activity, calcium sensitivity, and rate of myocyte contraction.

Adult rat cardiomyofibrils were phosphorylated *in vitro* by PKC isozymes and PKA using [γ-^{32}P] ATP. PKC isozymes and PKA showed intermolecular specificities in phosphorylating rat cardiac myofibrils. These results were substantiated by the *in situ* phosphorylation of adult rat cardiomyocytes using ^{32}P. Isolated cardiomyocytes were incubated with antagonists, agonist, inhibitors, or inducers of PKC isozymes and PKA

adrenergic pathways. These treatments resulted in increased and decreased phosphorylation of myofibrillar substrates, consequently supporting the *in vitro* data that displayed substrate specificity of PKC and PKA.

In general, phosphorylation of rat cardiac myofibrils by PKC isozymes (α, δ, ε, and ζ) and PKA decreased myofibrillar Mg^{2+}ATPase activity. However, they had diverse effects on calcium sensitivity. Phosphorylation of cardiomyocytes by PKC and PKA altered the velocity of contraction. The averages of the velocities were: control (untreated) -2.60×10^{-5} m/s, isoproterenol-6.00×10^{-5} m/s, phenylephrine- 3.47×10^{-5} m/s, TPA-1.67×10^{-5} m/s, and chlereythrine chloride-2.68×10^{-5} m/s.

In summary, these results revealed that PKC isozymes and PKA phosphorylation of rat cardiomyofibrils have specific substrates, and alternative biochemical and physiological effects on cardiac muscle contraction.

MODULATION OF CARDIAC PHYSIOLOGY IN ADULT RAT CARDIOMYOCYTES BY PROTEIN KINASE C AND PROTEIN KINASE A

A DISSERTATION

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LIST OF ABBREVIATIONS

mL ..... Milliliter

mM ..... Millimole

µL ..... Microliter

mg ..... Milligram

µg ..... Microgram

PKC ..... Protein Kinase C

PKA ..... Protein Kinase A

TnT ..... Troponin T

TnI ..... Troponin I

Ca^{2+} ..... Calcium

Mg^{2+} ..... Magnesium

CHAPTER 1

INTRODUCTION

With the rise in heart related illnesses and death, there is an increased need for research to yield a better understanding of cardiac function. At the close of 2004, the American Heart Association (AHA) reported that at least 79.4 million people in this country suffered from some form of cardiovascular disease (Rosamond et al. 2007, e69-e171). One person in four suffered from some form of high blood pressure, coronary heart disease, angina pectoris, myocardial infarction, stroke, rheumatic heart disease, congenital cardiovascular defects, or congestive heart failure. It has also been reported that heart disease is an equal opportunity killer; affecting all sexes, races, and ages. The AHA reported that in 2004, the number of male deaths from cardiovascular disease was 410,365 (47.1%), and the number of female deaths was 461,152 (52.9%). This is more than the next 16 causes of death combined. Though men commonly suffer from heart disease at a younger age than women, more than 50% of women alive today are expected to perish from cardiovascular disease. One in 3 men can expect to develop cardiovascular disease before the age of 60. For women of the same age, the odds are 1 in 10. As of 2007, the AHA also reports that cardiovascular associated diseases are anticipated to account for approximately 431.8 billion dollars in health care costs.

The goal of this project was to understand the biochemistry and functional physiology of contractile protein phosphorylation by PKC and PKA. The central

hypothesis was that *in situ* phosphorylation of contractile proteins by PKC and PKA results in alterations in biochemical and physiological phenomena. The involvement of PKC and PKA phosphorylation of contractile proteins, along with their ability to decrease Mg^{2+}ATPase activity and Ca^{2+} sensitivity, are considered to be vital parameters in modulating and evaluating cardiac muscle contractility and cardiomyopathy. It is hoped that the results of this project on the regulation of cardiac function will lead to new strategies that will prove useful in the treatment or deterrence of cardiovascular diseases.

SPECIFIC AIMS

Specific Aim 1: To characterize the *in vitro* phosphorylation of contractile proteins (TnI and TnT) in adult rat cardiomyofibrils by PKC isozymes and PKA. It was hypothesized that PKC isozymes and PKA will display site-specific phosphorylation of contractile proteins (TnT and TnI) in adult rat cardiomyofibrils. To investigate this hypothesis, the following procedures were employed:

A. Surgical removal of adult male rat heart and isolation of cardiomyofibrils.
B. Chromatographic purification of pan PKC and PKA from living tissue.
C. Phosphorylation of cardiomyofibrils by PKC isozymes and PKA using [γ-^{32} P] ATP.
D. Analysis of phosphorylated contractile proteins using scintillation counting, SDS-PAGE, autoradiography, and densitometry.

Specific Aim 2: To determine the modifications *in situ* phosphorylation of adult male rat cardiomyocytes using activators, inhibitors, agonists, or antagonists of PKC isozymes, PKA, and their respective adrenergic pathways. It was hypothesized that the *in situ*

phosphorylation of contractile proteins (TnT and TnI) in adult rat cardiomyocytes is modified by activators, inhibitors, agonists, or antagonists of PKC isozymes, PKA, and their respective adrenergic pathways. This hypothesis was investigated by the following procedures:

A. Surgical removal and perfusion of whole adult male rat heart.
B. Isolation of ventricular cardiomyocytes and microscopic analysis of viability.
C. *In situ* phosphorylation of cardiomyocytes using ^{32}P and treatments with activators, inhibitors, agonists, or antagonists of PKC isozymes, PKA, and their respective adrenergic pathways.
D. Analysis of *in situ* phosphorylated proteins by scintillation counting, SDS-PAGE, autoradiography, densitometry, graphing, and statistical evaluations.

Specific Aim 3: To determine the physiological changes associated with the phosphorylation of adult rat cardiomyofibrils with PKC isozymes and PKA. It was hypothesized that the phosphorylation of adult rat cardiomyofibrils with PKC isozymes and PKA will have alternating effects on the Mg^{2+}ATPase activity and calcium sensitivity. This hypothesis was investigated by using the following procedures:

A. Surgical removal and isolation of whole cardiomyofibrils from the heart of an adult male rat.
B. Enzymatic assays of Mg^{2+}ATPase activity at various Ca^{2+} concentrations.
C. Analysis of data using scintillation counting, graphing, and statistical evaluations

Specific Aim 4: To determine the mechanical changes associated with the *in situ.*

phosphorylation of adult rat cardiomyocytes by PKC isozymes and PKA using activators, inhibitors, agonists, or antagonists of PKC isozymes, PKA, and their respective adrenergic pathways. It was hypothesized that the in situ phosphorylation of adult rat cardiomyocytes by PKC isozymes and PKA using activators, inhibitors, agonists, or inducers of PKC isozymes, PKA, and their respective adrenergic pathways will display alternating effects on the cardiomyocytic contractile velocity. This hypothesis was investigated by the following procedures:

A. Surgical removal and perfusion of whole adult male rat heart.
B. Isolation of ventricular cardiomyocytes and microscopic analysis of viability.
C. Incubation of cardiomyocytes with activators, inhibitors, agonists, or inducers of PKC isozymes, PKA, and their respective adrenergic pathways.
D. Video recording of the cardiomyocyte contractile movements.
E. Measurement of distances and times between cardiomyocyte contractile phases, and calculation of velocities.

CHAPTER 2

LITERATURE REVIEW

CARDIAC MUSCLE CONTRACTION

The well-known function of the heart involves its contraction and relaxation to pump essential nutrients contained in the blood throughout the body. The heart is made up of several muscle cells, termed cardiomyocytes, which work together as one large muscle. The contraction of the heart is spontaneously initiated by the heart muscle itself rather than from an external stimulus, a phenomenon known as autorhythmicity. Autorhythmic cells of the heart, localized at the sinoatrial nodes and purkinje fibers, are specialized for initiating and conducting the action potentials responsible for contraction of the contractile cells of the heart. The membrane potential of cardiomyocytes drifts until a threshold is reached. At this point, the membrane fires and triggers an action potential. The action potential in the contractile cells travel down transverse tubules (T-tubules), simultaneously releasing Ca^{2+} from the sarcoplasmic reticulum and stimulating the entry of Ca^{2+} from the extracellular fluid (ECF). Once the calcium enters the cytosol, it binds to the troponin C (TnC) (18kD) component of the troponin-tropomyosin complex. This stimulates an increase in affinity of the actomyosin Mg^{2+}ATPase inhibitory component, troponin I (TnI) (23kD), for TnC, while causing a decrease in TnI affinity for actin (Ward et al. 2002, 41795-41801; Dong et al. 1999, 31382-31390; Gordon et al. 2000, 853-924). The troponin T (TnT) (37kD) component (bound to TnC,

TnI, and tropomyosin (Tm) (33kD)) pulls Tm away from its blocking position between actin and the myosin head cross bridge. This exposes the Mg^{2+}ATPase molecules of actin to the ATP bound molecule of the myosin head cross bridge, resulting in a power stroke which pulls actin inward along the myosin filament, otherwise known as muscle contraction.

ADRENERGIC PATHWAYS AND KINASE ACTIVATION

Cardiac muscle contraction may be influenced by the autonomic nervous system. Epinephrine (from the adrenal gland) and norepinephrine (from the postganglionic sympathetic nerves) are released in response to stress. β_1- and β_2- adrenergic receptors (located on the surface of myocytes) receive these hormones, which pass through a cascade of signal transduction events that include the guanine nucleotide-binding regulatory proteins and effector enzyme, adenylate cyclase (Zhu et al. 2005, 507–509; Kuschel et al. 1999, 22048-22052; Feldman et al. 1993, 27-34). This signaling cascade results in the production of cyclic AMP, activation of protein kinase A (PKA), and ultimately the phosphorylation of myofibrillar proteins (i.e. the troponin complex) that are essential to the excitation or inhibition of muscle contraction (Zhang et al. 1995, 30773-30780). In chronic heart failure, positive inotropic effects of β-adrenergic receptor agonists are significantly reduced due to loss of receptor functions (receptor uncoupling) and reduction in the number of receptors (down regulation) (Wang et al. 2005, H151-H159; Ungerer et al. 1993, 454-463).

On the other hand, the α-adrenergic receptor is the means by which the signaling protein, protein kinase C (PKC) is induced (Price et al. 2002, 9570–9579). It has been

indicated to play an important role in cardiac function (Puceat et al. 1994, 249-268). The regulation of contraction through the α-adrenoreceptor pathway includes the second messenger diacylglycerol (DAG) and the phosphoinositol (PI) derivative, inositol 1, 4, 5-triphosphate (IP_3). Certain PKC isozymes are activated by DAG (Pi et al. 1997, 92-100) while IP_3 stimulates the release of sequestered Ca^{2+} (stored in the sarcoplasmic reticulum of the muscle cell), which activates Ca^{2+}-dependent PKC isozymes. These isozymes are the classical or conventional subtypes (α, β_I, β_{II}, and γ) and are also phosphatidylserine (PS)-dependent. Other types of isozymes include the novel subtypes (δ, ε, η, and θ) that are Ca^{2+}-independent, but PS/diacylglycerol-dependent, and the atypical subtypes (ζ, λ, and ι) that are PS-dependent, but Ca^{2+}- and diacylglycerol-independent (Nishizuka 1992, 8808-8814). Effects of PKC on cellular functions depend, therefore, on the properties of the isozymes. These effects include: (a) cellular and tissue distribution, (b) response to stimuli, (c) substrate specificity, and (d) activation pathway. Stimulation by α_1-adrenergic agonists causes PKC to translocate into myofibrillar compartments of the heart (Korzick et al. 2001, H581 - H589; Otani et al. 1992, 22-26; Mochly-Rosen et al. 1992, 693-706; Liu et al. 1989, 1105-1110), suggesting that its target substrates are most likely to be found there. This eventually led to finding the subcellular localization of six PKC isozymes (α, β_I, β_{II}, δ, ε, and ζ) in non-stimulated cardiac myocytes and in myocytes stimulated by noreprinephrine or phorbol 12-myristate 13-acetate (Disatnik et al. 1994, 287-297).

Receptor uncoupling involves phosphorylation of receptor proteins by specific α-adrenergic receptor kinases. In failing myocardium Ca^{2+} sensitivity of isometric

contraction is increased, but PKC and PKA phosphorylation of contractile proteins (TnI and TnT) decreases Ca^{2+} sensitivity (Suematsu et al. 2001, H637-H646; Wolff et al. 1995, 781-789), indicating a regulatory role of phosphorylation of these proteins in cardiac function. Several signal transduction defects, such as reduction in cAMP formation and an altered intracellular Ca^{2+} handling, have been observed in human cardiomyopathy (Saucerman and McCulloch 2006, 348-361; Bohm et al. 1994, 1713-1719). Treatment of myofibrils from chronic heart failure with PKA decreases Ca^{2+} sensitivity (Hahn et al. 2003, 1111-1119; Knowlton et al. 1993, 15374-15380). It is possible that cAMP-dependent PKA and Ca^{2+}-dependent PKC functions will consequently be altered in the pathologic heart. Therefore, PKA and PKC phosphorylation of TnI and TnT is a vital regulatory parameter for evaluating contractile processes in the heart.

All the contractile proteins of the heart have been implicated in one or more forms of cardiomyopathy. For example, familial hypertrophic cardiomyopathy (FHC) has been linked to modified cardiac TnT (Frey et al. 2006, 29575-29582; Thierfelder et al. 1994, 701-712). A mutation in the splice donor of intron 15 of the TnT gene causes FHC. FHC has been associated with several mutations in the gene encoding human cardiac troponin I (HCTnI), in which case a missense mutation in the inhibitory region of TnI replaces an arginine residue (position 145) with a glycine and cosegregates with the disease (Lang et al. 2002, 11670-11678). Recent findings have supported the hypothesis that thin filament heterogeneity, due to the co-expression of alternatively spliced cTnT variants, may desynchronize myocardial contraction and contribute to the pathogenesis and

pathophysiology of cardiomyopathy and heart failure (Biesiadecki et al. 2002, 50275-50285). Experiments have shown that in dilated cardiomyopathy and heart failure, the organization and the amount of contractile proteins (actin, TnI, TnT, and Tm) suffers severe disarrangement or lack of myofilaments (Venkatraman et al. 2003, 41670-41676; Hein et al. 1994, 1291-1306). It has also been reported that mutation on TnT accounts for about 15% of all cases of FHC. Findings have shown that *in vitro* phosphorylation of contractile components by PKC affect interactions between the thick and thin filament, displaying an inhibition of Ca^{2+}-stimulated Mg^{2+}ATPase activity and alterations in calcium sensitivity (Jideama et al. 2006, 1-9; Noland et al. 1995, 25445-25454). Though there have been many advances in the understanding of contractile phosphorylation by PKC and PKA, few have extended beyond *in vitro* experiments.

CHAPTER 3

MATERIALS AND METHODS

PREPARATION OF RAT CARDIAC MYOFIBRILS FOR *IN VITRO* STUDIES

Surgical removal of the heart was performed on adult male Sprague-Dawley strain rats. The heart was cleaved from the body of the rat by severing the vena cava and aortic vessels. Myofibrils were extracted from rat ventricular myocardia by detergent extraction as described by published methods (Murphy et al. 1990, 46-49). Rat ventricles were minced and homogenized in 10-15 mL of homogenate buffer (25mM imidazole at pH 7.0 and 2-mercaptoethanol) on ice. The homogenate solution was diluted (2 fold) with solution A (60 mM KCl, 30 mM imidazole at pH 7.0, 15 mM mecarptoethanol, and 2.5 mM $MgCl_2$) and centrifuged at 10,000 times gravity (g) for 20 minutes in a J2-21 Beckman centrifuge. The pellet was retrieved and resuspended in solution A (5 mL/g) and centrifuged at 5,000 × g for 10 minutes. The resulting pellet was resuspended in solution A with 1 mM EGTA and centrifuged at 5,000 × g for 10 minutes. The resulting pellet was resuspended in solution A with 1% Triton X-100 and centrifuged at 5,000 × g for 10 minutes. The pellet was resuspended in solution B that contained 60 mM KCl, 30 mM imidazole at pH 7.0, and a protease inhibitor cocktail (0.01mg/mL leupeptin, 0.01mg/mL antipain, 0.01mg/mL aprotinin, and 0.01mg/mL pepstatin A). The resuspended was then centrifuged at 5,000 × g for 10 minutes. The pellet was resuspended and pelleted in solution B two more times. The resulting pellet was

resuspended in ice cold ethanol, and centrifuged at 5,000 × g for 10 minutes. The pellet was resuspended in 50 mL of polypropylene and centrifuged at 5,000 × g for 10 minutes. The pellet was subjected to the same polypropylene resuspention and centrifugation twice more. The resulting pellet was resuspended in an ice-cold solution consisting of acetone and the protease inhibitor cocktail for 2 hours.

PURIFICATION AND ASSAY OF KINASE ENZYMES

Pan PKC was purified from bovine brain extracts by DEAE-cellulose and Affi-Gel Blue chromatography as performed in previously published methods (Uchida et al. 1984, 12311-12314). The enzyme was further purified to homogeneity by means of PCPA affinity chromatography. PKA was purified from bovine heart extracts using anion-exchange chromatography according to previously published methods (Kuo et al. 1970, 79-91). PKC isozymes α, δ, ε, and ζ were purchased from Upstate/Millipore Corporation (Charlottesville. VA, USA). Protein kinase activity was assayed in a 200 μL (pH 7.5) reaction mixture (0.05 mL PIPES, 0.02mL $MgCl_2$, 0.01mL phosphatidylserine, 0.02 mL of H1histone, 0.05 mL distilled water, 0.02 mL of enzyme, 0.02 mL EGTA (2mM)/ $CaCl_2$ (5mM), and 0.01 [γ^{32}P] ATP) and incubated for 5 minutes in a 30^0C water bath shaker (ATP was used to initiate the reaction). Phosphatidylserine and diolein were combined, evaporated under nitrogen, and dispersed in water or 10 mM Tris (pH 7.5) by sonication (60 Sonic Demembrator, Fisher Scientific) for 2-5 minutes. Reactions were terminated by the addition of 1 mL of ice cold 5% trichloroacetic acid (TCA) and 0.2 mL of 0.65%bovine serum albumin (BSA). Precipitated protein was sedimented by centrifugation, redissolved in 0.5 N NaOH, reprecipitated with stopping solution. This

was repeated three times and finally counted in a LS6500 Beckman scintillation spectrometer.

IN VITRO PHOSPHORYLATION OF RAT VENTRICULAR MYOFIBRILS

Phosphorylation of rat ventricular myofibrils by classical isozyme PKC-α was carried out according to published methods (Jideama et al. 1996, 23277-23289; Venema et al. 1993, 401-406; Noland et al. 1993, 53-65) using 0.2 mL of the reaction mixture containing 50 mM Tris/HCl (pH 7.5), 100 mM $MgCl_2$, 25 μg/ml of PS, 2 μg/ml of diolein, 100 mM $CaCl_2$, 100 mM KCl, 30 mM 2-mercaptoethanol, 10 nM calyculin A, 21 μg of myofibrils, and appropriate amounts of enzyme. Phosphorylation of myofibrils by novel (PKC-δ and -ε) and atypical (PKC-ζ) isozymes was performed in the same manner as classical isozymes, except for omitting the use of $CaCl_2$ in novel reaction mixtures and $CaCl_2$/diolein in atypical reaction mixtures. Phosphorylation by PKA was carried out in the same manner as PKC phosphorylation with the exception of 10 mM cyclic AMP being added as an activator instead of phospholipid, diolein, and $CaCl_2$.

The reaction was initiated by the addition of 5 μM [γ-^{32}P] ATP (1-2 x 10^6 cpm) and incubated for 60 minutes at 30°C. It is likely that actomyosin ATPase and PP1 may be present in the myofibril preparation, therefore, repeated additions of 5 μM [γ-^{32} P] ATP were used for stoichiometric phosphorylation. The ^{32}P- myofibrils were subjected to direct precipitation with 5% trichloroacetic acid containing tungstate. Bovine serum albumin was used as a carrier protein during precipitation of myofibrils. The phosphorylation of samples was evaluated by SDS-PAGE, autoradiography, densitometry (*UN-SCAN-IT gel*™ gel and graph digitalizing software from the Silk Scientific

Corporation (version 6.1)), and liquid scintillation counts.

PROTEIN SEPARATION AND ANALYSIS

Phosphorylated cardiomyofibrils and cardiomyocytes were analyzed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) as performed by published methods (Laemmli 1970, 680-685). Phosphorylated proteins were boiled for 5 minutes and loaded in the wells of a 12% SDS-PADE gel with a molecular weight (MW) marker lane. The proteins and MW markers were separated using a voltage of 25mV. This was followed by staining with Coomassie Blue R-250 (Sigma-Aldrich, USA) and destaining using a 50% methanol, 10% acetic acid solution. The gel was dried using a 543 BioRad gel dryer. The gels were placed in Kodak cassettes with Kodak BioMax MS Film (VWR, USA) for varied times based on the strength of the radioisotope as determined by scintillation counts using a LS6500 Beckman scintillation spectrometer. The autoradiograph films were exposed using a Kodak M35 XOMAT processor and further analyzed by the*UN-SCAN-IT gel*™ gel and graph digitalizing software from Silk Scientific Corporation (version 6.1) in order to determine the integrated density values of the bands. Graphs and statistical analysis were formulated by using the GraphPad Prism® software (version 4.03).

ISOLATION OF RAT CARDIAC MYOCYTES FOR *IN SITU* STUDIES

A modified version of a previously published method was used (Murphy et al. 1990, 46-49). The heart of an adult male Sprague-Dawley strain rat was surgically extracted by cleaving the vena cava and aortic vessels, and immediately placed in cold HEPES buffer at pH 7.0 containing 4.9 mM KCl, 137 mM NaCl, 20 mM Hepes, 1.2 mM NaH_2PO_4, 15

mM dextrose, and 1.2 mM $MgSO_4$. Perfusion of the heart was conducted by way of the aorta. The heart was purfused with Earles Balanced Salt Solution (EBSS) (5.4 mM KCl, 116.4 mM NaCl, 26.2 mM $NaHCO_3$, 1.0 mM NaH_2PO_4 x H_2O, 5.6 mM dextrose, and 1.6 mM $MgSO_4$), at 37^0C, until the solution eluting from the heart was clear in color. EBSS was mixed with 1% collagenase B (Worthington Biochemical Corporation) and 0.01mg/mL protease inhibitors. The EBSS/collagenase solution was perfused through the heart at 37^0C for 15 –20 minutes or until the heart became uniformly lighter in color. The heart was then perfused with EBSS mixed with 1% bovine serum albumin (BSA) for 10 minutes to wash away excess collagenase. HEPES buffer with 1% BSA was perfused through the heart with increasing concentrations of $CaCl_2$ (0.25 and 0.5) for 10 minutes each. This was followed by perfusion with HEPES buffer (without BSA) that had a $CaCl_2$ concentration of 1.0 mM for 10 minutes. The ventricle of the heart was removed, placed in a small beaker containing HEPES buffer (1.0 mM $CaCl_2$) in a water bath at 37°C, and diced into minute particles. The resulting myocyte suspension was filtered with nylon gauze. The viability of the isolated cardiomyocytes was determined by observation using a trinoculor microscope with a CCD camera. Only preparations yielding cardiomyocytes that were 70% or better in viability (determined by their elongated/rod shape and contractile movement) were used in further experimentation.

IN SITU PHOSPHORYLATION OF RAT VENTRICULAR MYOFIBRILS

In situ phosphorylation of cardiomyocytes was performed using a modified version of a previously published method (Jideama et al. 1996, 23277-23289). Isolated cardiomyocytes were incubated (1.5 hour at 37° C) in phosphate free Dulbecco's

Modified Eagle's Medium (DMEM) containing ^{32}P. Following incubation, 1 mL aliquots of the cells underwent additional incubation (10 minutes at 37° C) with an α-adrenergic agonist (5 mM phenylephrine), a β-adrenergic agonist (100 nM isoproterenol), a PKC activator (10 mM 12-10-tetradecanolyphorbol-13-acetae (TPA)), a Ca^{2+}-dependent PKC inhibitor (2.0 mM Gö6976), a PKC inhibitor (600 nM chelerythrine chloride), or a β-adrenergic antagonist (30 mM prazosin). Phenylephrine and isoproterenol were used to indicate the involvement of the PKC and PKA pathways, respectively. After the various treatments, the samples were pelleted and washed in buffer A solution (1mM PMSF, 100 μg/mL leupeptin, 100 μg/mL antipain, 100 μg/mL pepstatin A, 0.1 mM NaOV, 100 nM CCA, and 5 mM EGTA), sonicated in buffer B solution (50 mM KH_2PO_4, 70 mM NaF, 5 mM EDTA, 1% Triton X-100, 100 μg/mL leupeptin, 100 μg/mL antipain, 100 μg/mL pepstatin A, and 100 nM CCA), iced for 30 minutes, and washed in modified buffer B solution (without Triton X-100). The phosphorylation of samples was evaluated by SDS-PAGE, autoradiography, densitometry (*UN-SCAN-IT gel*™ gel and graph digitalizing software from the Silk Scientific Corporation (version 6.1)), and liquid scintillation counts. Graphs and statistical analysis were formulated by using GraphPad Prism® software (version 4.03).

DETERMINATION OF Ca^{2+}-STIMULATED Mg^{2+} ATPASE ACTIVITY AND Ca^{2+} SENSITIVITY OF MYOFIBRILS

Ca^{2+}-stimulated Mg^{2+}ATPase activity was assayed according to published methods (Jideama et al. 2006, 1-9; Noland et al. 1993, 53-65). The assay was carried out at 30°C for 10 minutes in standard reaction mixtures (0.5 mL) containing 21 μg of rat

cardiac myofibrils, 10 mM MOPS-KOH (pH 7.0), 4.1 mM Mg^{2+}acetate, 2.1 mM [γ-^{32}P] ATP (5-8 x 10^6 cpm), 1 mM DTT, 1 mM EGTA, and 1.05 mM $CaCl_2$. $CaCl_2$, when present, was added as a mixture with EGTA and MOPS-KOH to provide a calculated free Ca^{2+}-concentration of 0.01-100 mM in the assay mixture. KCl was added to bring the final ionic strength to 18 mM. The reactions were initiated by addition of radioactive [γ-^{32}P] ATP, terminated by addition of 10% charcoal suspension, and the released ^{32}Pi was determined as previously reported (Noland et al. 1995, 25445-25454). Stability constants used in calculating the total concentration of reaction constituents required to give the necessary free Ca^{2+} concentrations, were determined using previously published procedures (Fabiato et al. 1988, 469-495). The EC_{50} value for Ca^{2+} activation was calculated from the equilibrium constant *(K')* according to formula: $EC_{50} = K^{(1/nh)}$ (where *nh* is the Hill coefficient). Graphs and statistical analysis were formulated by using GraphPad Prism® software (version 4.03).

DETERMINATION OF VELOCITY OF MYOCYTE CONTRACTION

Surgical removal and perfusion of adult male Sprague-Dawley strain rat hearts were conducted as mentioned in the prior section of the Materials and Methods. The isolated cardiomyocytes were examined for adequate viability and incubated in isoproterenol, phenylephrine, TPA, or chelerythrine chloride for 10 minutes at 37°C. The treated myocytes were then placed on slides and observed using a trinocular microscope with a NAVCO® CCD color camera and the Asymetic Digital Video Producer 5.0® software program. The video of contracting myocytes was played back and stopped as the cardiomyocyte reached its maximum contracted length and again stopped at its

maximum relaxed length. The measurements were recorded along with their respective times. These values (distance and time) were used to calculate the velocity of contracting myocytes. The values were expressed as the mean of three different experiments. Graphs and statistical analysis were formulated by using GraphPad Prism® software.

CHAPTER 4

RESULTS

IN VITRO SUBSTRATE SPECIFIC PHOSPHORYLATION OF RAT CARDIAC MYOFIBRILS

Determining substrate specificity can serve as a vital parameter in understanding the function of a particular enzyme in cardiac function. In order to determine the substrate specificity of contractile component associated enzymes, rat ventricular myofibrils were phosphorylated by PKC isozymes (α, δ, ε, and ζ) and PKA using [γ-^{32}P] ATP. It was observed that PKC isozymes (α, δ, ε, and ζ) and PKA expressed in adult rat cardiomyofibrils showed distinct substrate specificities in phosphorylating contractile components (Figure 1). Our results indicated that in intact rat ventricular cardiomyofibrils: pan PKC (a mixture of the PKC isozymes) phosphorylated TnT 1.65 times more than TnI; PKC-α phosphorylated TnT 1.62 times more than TnI; PKC-δ phosphorylated TnI 1.05 times more than TnT; PKC-ε phosphorylated TnT 1.12 times more than TnI. PKC-ζ phosphorylated TnT 1.78 times more than TnI; and PKA phosphorylated TnI 12 times more than TnT. These results confirmed that PKC isozymes and PKA display different substrate affinities in phosphorylating TnI and TnT in intact cardiac myofibrils. PKC-ε phosphorylated proteins at 83 kDa, as determined by the relative mobility of the standard molecular weight marker (Figure 1). This molecular

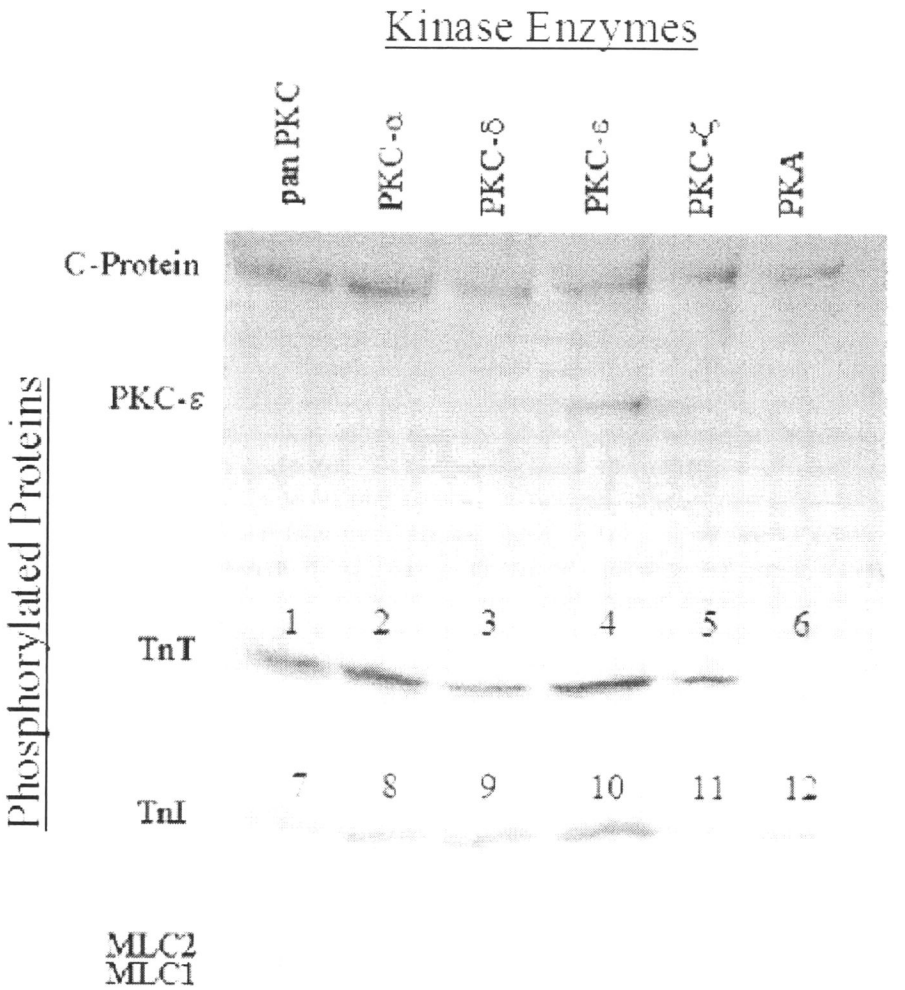

Figure 1. Autoradiograph showing substrate specificities of PKC isozymes and PKA for *in vitro* phosphorylation of adult rat cardiomyofibrillar proteins. The intergrated density values were (1) 30600, (2) 33000, (3) 22200, (4) 34300, (5) 19200, (6) 1200, (7) 18600, (8) 20400, (9) 21200, (10) 30600, (11) 10800, and (12) 14400.

weight is approximately the same as that of this isozyme. Therefore, PKC-ε is most likely responsible for the autophosphorylation of PKC.

CONCENTRATION-DEPENDENT PHOSPHORYLATION OF RAT CARDIAC MYOFIBRILS

Certain conditions must be taken into consideration when investigating with the use of kinase enzymes. Enzymatic activity may be altered by conditions such as the temperature and pH of the environment, as well as the enzyme to substrate concentration ratio. Previous reports (Noland et al. 1995, 25445-25454) have indicated that in reconstituted troponin (Tn), PKC-α phosphorylated TnI more than TnT at concentrations less than 20 μM. At concentrations above 20 μM, TnI exhibits substrate inhibition to this isozyme. Therefore, in support of specific aim 1(characterization of the *in vitro* phosphorylation of contractile proteins (TnI and TnT) in adult rat cardiomyofibrils by PKC isozymes and PKA), it was of interest to further characterize the biochemical modifications of contractile components (TnI and TnT) associated with the concentration-dependent *in vitro* phosphorylation of intact adult rat cardiomyofibrils using PKC-α. Concentration-dependent phosphorylation of rat cardiac myofibrils by PKC-α showed that TnI is phosphorylated more than TnT at lower substrate (myofibril) concentrations of (Figure 2), as confirmed by densitometry. Contrarily, at higher substrate concentrations, TnT is phosphorylated more than TnI. This implies that PKC-α phosphorylation of the myofibrils shows substrate inhibition of TnI at higher substrate concentrations. This TnI substrate inhibition of PKC-α (Figure 2) is believed to have a significant physiological impact on cardiac function, as will be addressed in the

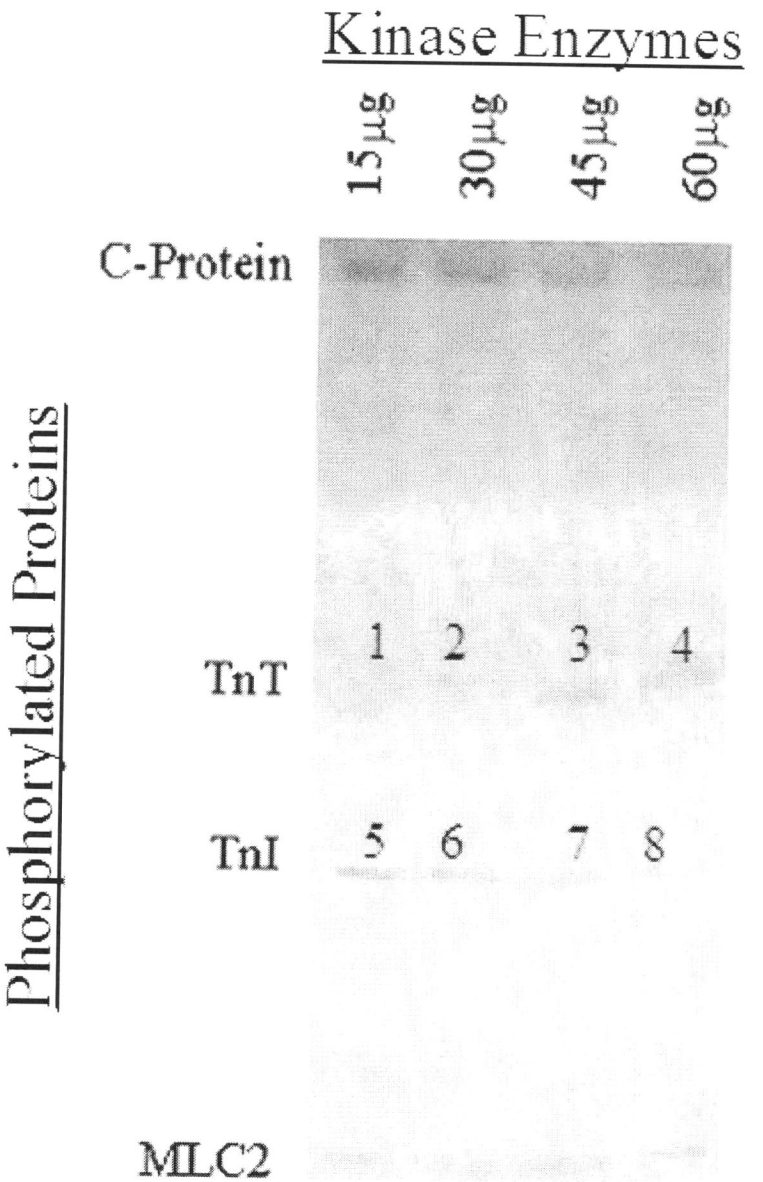

Figure 2. Concentration-dependent phosphorylation by PKC-α showing substrate inhibition of TnI in rat ventricular myofibrils. The intergrated density values were (1) 4914, (2) 5460, (3) 7644, (4) 7644, (5) 8736, (6) 7644, (7) 7098, and (8) 6006.

discussion component of this document.

IN SITU PHOSPHORYLATION OF RAT CARDIOMYOCYTES

One of the primary goals of biomedical research is for one's research to eventually be examined *in vivo* (within the living organism). Before this level of research is reached there are usually intermediate sets of experimentation conducted *in situ* (within a living isolated cellular environment). Therefore, it was of interest to compare results that were obtained by way of the *in vitro* (within an artificial environment) experiments to results that resulted from *in situ* experimentation. *In situ* phosphorylation of isolated adult rat cardiomyocytes was used to substantiate the *in vitro* experiments, which indicated site-specific phosphorylation of cardiomyocytes. One-milliliter aliquots of isolated cardiomyocytes (Figure 3) incubated (1.5 hours) with ^{32}P, were subjected to further incubations (10 minutes) with phenylephrine (α-adrenergic agonist), isoproterenol (β-adrenergic agonist), or 12-10-tetradecanolyphorbol-13-acetae (TPA) (diacylglycerol-dependent PKC activator). The identity of the phosphorylated proteins was based on the comparison of their relative migration with a standard protein molecular weight, and confirmed by autoradiography and densitometry. The autoradiograph of the SDS-PAGE gel indicated that overall, TnT was phosphorylated to a greater extent than TnI (Figures 4, 5a, and 5b; Table 1). It was observed that when the signaling pathway of PKC (α-adrenergic signaling transduction pathway) was stimulated with phenylephrine, TnT's phosphorylation was increased (1.12 fold increase (1.60×10^5 intensity units (iu))), while TnI phosphorylation also increased (1.19 fold (0.64×10^5 iu)), as compared to respective controls (1.43×10^5 iu for control TnT and 0.54×10^5 iu for control TnI) (Figures 4, 5a,

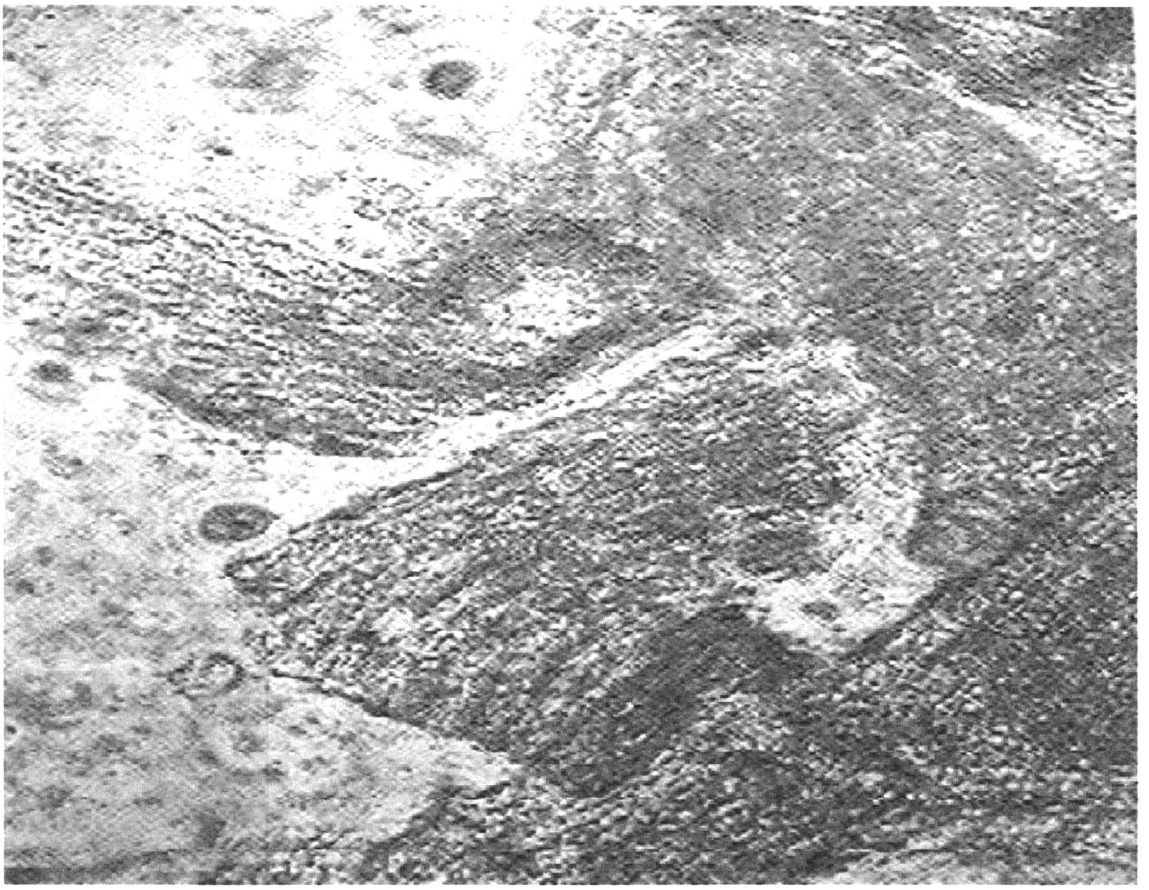

Figure 3. Isolated adult rat cardiomyocytes at 200x. Only preparations displaying 70% viability or better were used in further experimentation.

and 5b; Table 1). The results obtained from *in situ* phosphorylation due to the induction of PKA by the beta-adrenergic pathway using isoproterenol indicated a decrease (1.30 fold decrease (1.10×10^5 iu)) in TnT phosphorylation, and the greatest phosphorylation increase of TnI among the treatments (2.07 fold increase (1.12×10^5 iu)). The activation of diacylglycerol (DAG)-dependent PKC isozymes by TPA yielded an approximately 1.05 fold increase in TnT phosphorylation (1.50×10^5 iu) and a 1.17 fold decrease in the phosphorylation of TnI (0.46×10^5 iu), as compared to the respective controls.

Additionally, it was of interest to examine the effect of inhibiting Ca^{2+}-dependent PKC isozymes, all PKC isozymes, or the α-adrenergic pathway. Therefore, isolated ^{32}P-myocytes were incubated for 10 minutes with Gö6976 (Ca^{2+}-dependent PKC inhibitor), chelerythrine chloride (nonselective PKC inhibitor), or prazosin (α-adrenergic antagonist). Myocytes treated with Gö6976 showed a slight increase of 1.06 fold (0.57×10^5 iu) in the phosphorylation of TnI, as well as a slight increase of 1.08 fold (1.54×10^5 iu) in the phosphorylation of TnT (Figures 4, 5a, and 5b; Table 1). Myocytes treated with chelerythrine chloride showed a nearly significant decrease of 1.36 fold (1.05×10^5 iu) in the phosphorylation of TnT, and a slight increase of 1.19 fold (0.64×10^5 iu) in the phosphorylation of TnI. Myocytes treated with prazosin showed a significant decrease of 1.55 fold (0.92×10^5 iu) in the phosphorylation of TnT, and a moderate, though non-significant, decrease of 1.17 fold (0.46×10^5 iu)in the phosphorylation of TnI.

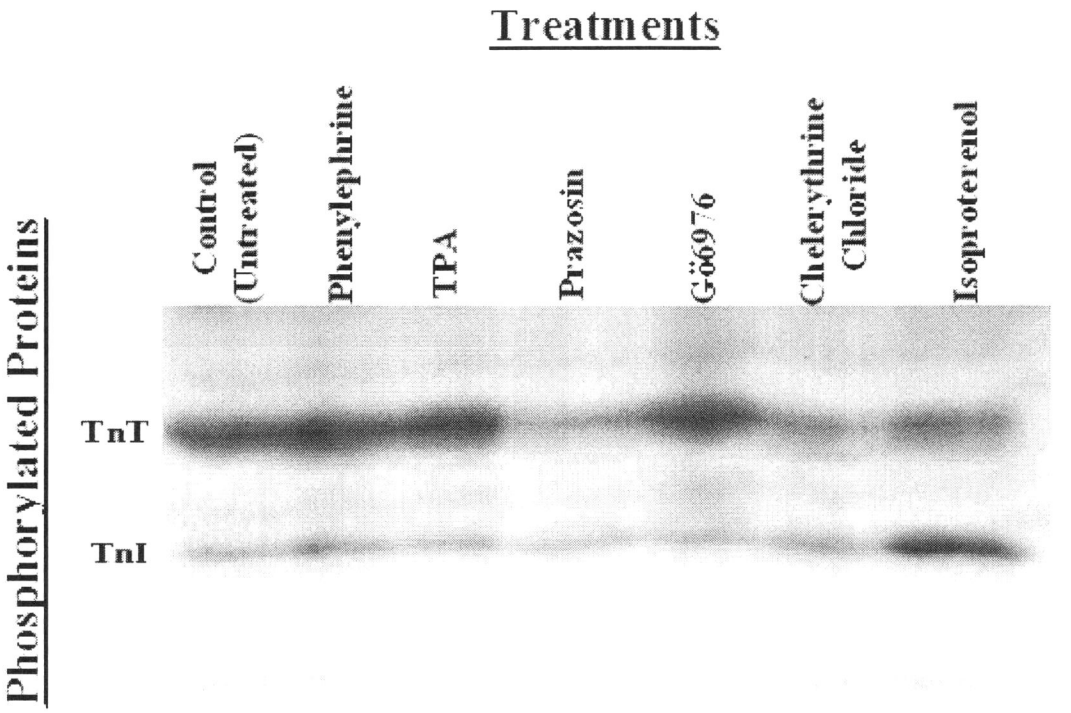

Figure 4. Autoradiograph of the *in situ* phosphorylation of adult rat cardiomyocyte contractile proteins. Aliquots of isolated cardiomyocytes incubated with ^{32}P, were subjected to further incubations with agonists, antagonists, activators, or inhibitors of PKC or PKA. The samples were subjected to SDS-PAGE, followed by autoradiography and densitometry. The values were expressed as the mean of three different experiments.

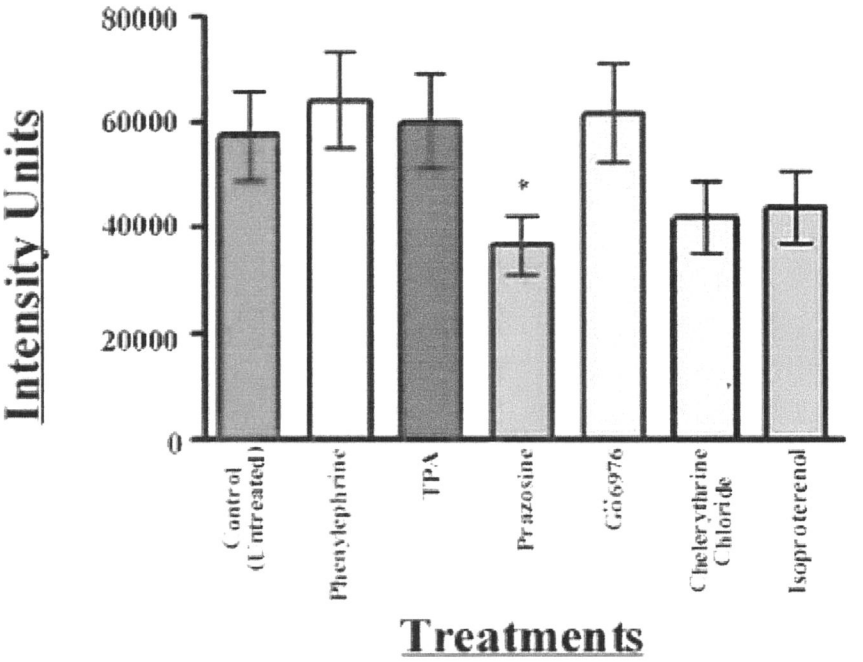

Figure 5a. Densitometry values for the autoradiogragh of the *in situ* phosphorylation of adult rat cardiomyocyte contractile protein, TnT (Figure 4). Aliquots of isolated cardiomyocytes incubated with ^{32}P, were subjected to further incubations with isoproterenol, phenylephrine, or TPA. The samples were subjected to SDS-PAGE, followed by autoradiography and densitometry (measured in intensity units). The values were expressed as the mean of three different experiments. The * indicates values significantly different from the control.

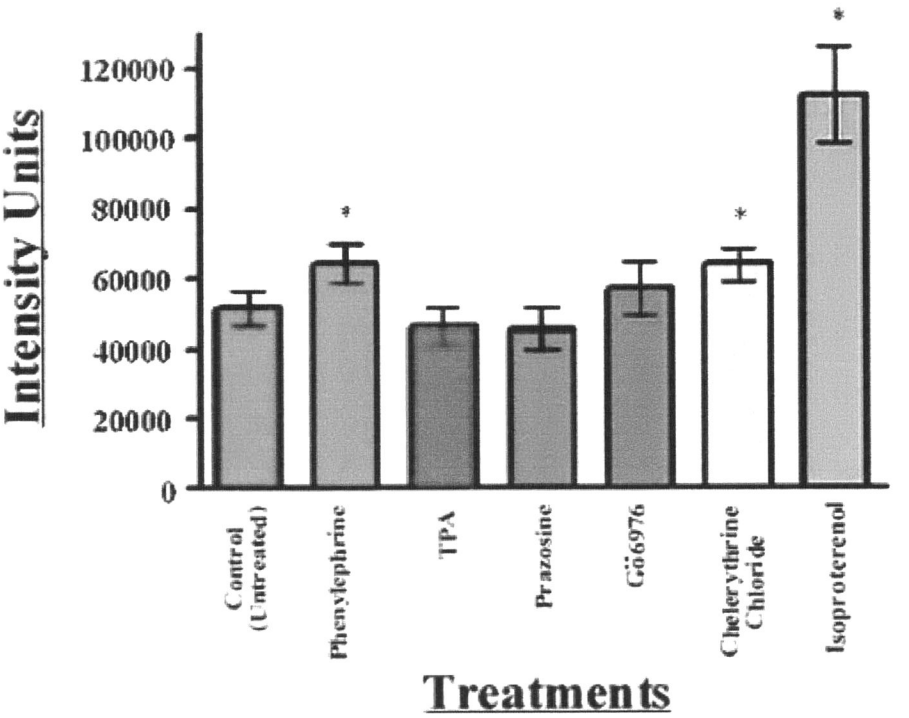

Figure 5b. Densitometry values for the autoradiogragh of the *in situ* phosphorylation of adult rat cardiomyocyte contractile protein, TnI (Figure 4). Aliquots of isolated cardiomyocytes incubated with ^{32}P, were subjected to further incubations with isoproterenol, phenylephrine, or TPA. The samples were subjected to SDS-PAGE, followed by autoradiography and densitometry (measured in intensity units). The values were expressed as the mean of three different experiments. The * indicates values significantly different from the control.

Table 1. Densitometry values for the autoradiogragh of the *in situ* phosphorylation of adult rat cardiomyocyte proteins, TnT and TnI (Figure 4).

	Control (Untreated)	Gö6976	Chelerythrine Chloride	Isoproterenol
TnT	$1.43\times10^5\pm0.21$	$1.54\times10^5\pm0.23$	$1.05\times10^5\pm0.17$	$1.10\times10^5\pm0.17$
TnI	$0.54\times10^5\pm0.05$	$0.57\times10^5\pm0.07$	$0.64\times10^5\pm0.04$	$1.12\times10^5\pm0.14$
	Control (Untreated)	**Phenylephrine**	**TPA**	**Prazosin**
TnT	$1.43\times10^5\pm0.21$	$1.60\times10^5\pm0.23$	$1.51\times10^5\pm0.22$	$0.92\times10^5\pm0.14$
TnI	$0.54\times10^5\pm0.05$	$0.64\times10^5\pm0.05$	$0.46\times10^5\pm0.05$	$0.46\times10^5\pm0.05$

Aliquots of isolated cardiomyocytes incubated with ^{32}P, were subjected to further incubations with agonists, antagonists, activators, or inhibitors of PKC or PKA. The samples were subjected to SDS-PAGE, followed by autoradiography and densitometry (measured in intensity units). The values were expressed as the mean of three different experiments.

Ca^{2+}-STIMULATED Mg^{2+}ATPASE ACTIVITY AND Ca^{2+} SENSITIVITY OF RAT CARDIAC MYOFIBRILS

As previously discussed, the troponin complex regulates the contraction of cardiac muscle. One of the most important steps in contraction involves the binding of calcium to the TnC component of the troponin complex. The more sensitive the complex is to calcium, the more likely contraction is to occur. Additionally, contraction results from the interaction of actin and myosin, whereby ATP (bound to the myosin head) is hydrolyzed by Mg^{2+}ATPase of the actin molecule. Therefore, it is reasonable to associate the level Mg^{2+}ATPase activity with the amount of contraction that occurs. Phosphorylation of contractile components by different kinases is likely to alter the level of Ca^{2+}sensitivity and Mg^{2+}ATPase activity. With this in mind, the physiological relevance of the phosphorylation of ventricular cardiomyofibrils by PKC isozymes (α, δ, ε, and ζ) and PKA was examined (Figure 6). The results were summarized (Table 2) in order to determine the effects of PKC isozymes and PKA phosphorylation on myofibrillar Mg^{2+}ATPase activity and Ca^{2+} sensitivity. In general, it was found that phosphorylation of rat ventricular cardiomyofibrils by PKC isozymes (α, δ, ε, and ζ) and PKA decreased myofibrillar Ca^{2+}-stimulated Mg^{2+}ATPase maximal activity (Figure 6). However, they have variable effects on myofibrillar Ca^{2+}sensitivities (Table 2). Phosphorylation of rat cardiac myofibrils by PKC-δ, PKC-ε, and PKA fairly decreased myofibrillar Ca^{2+} sensitivity (5.0 μM, 5.3 μM, 5.0 μM, respectively), when compared to the control (no enzyme) (3.2 μM). Whereas PKC-α phosphorylation of cardiac myofibrils greatly decreased Ca^{2+} sensitivity (16.5 μM), phosphorylation of these

cardiomyofibrils by PKC-ζ sharply increased Ca^{2+} sensitivity (1.9 μM). Therefore, PKC-α has implications to be functionally antagonistic to PKC-ζ in cardiac physiology.

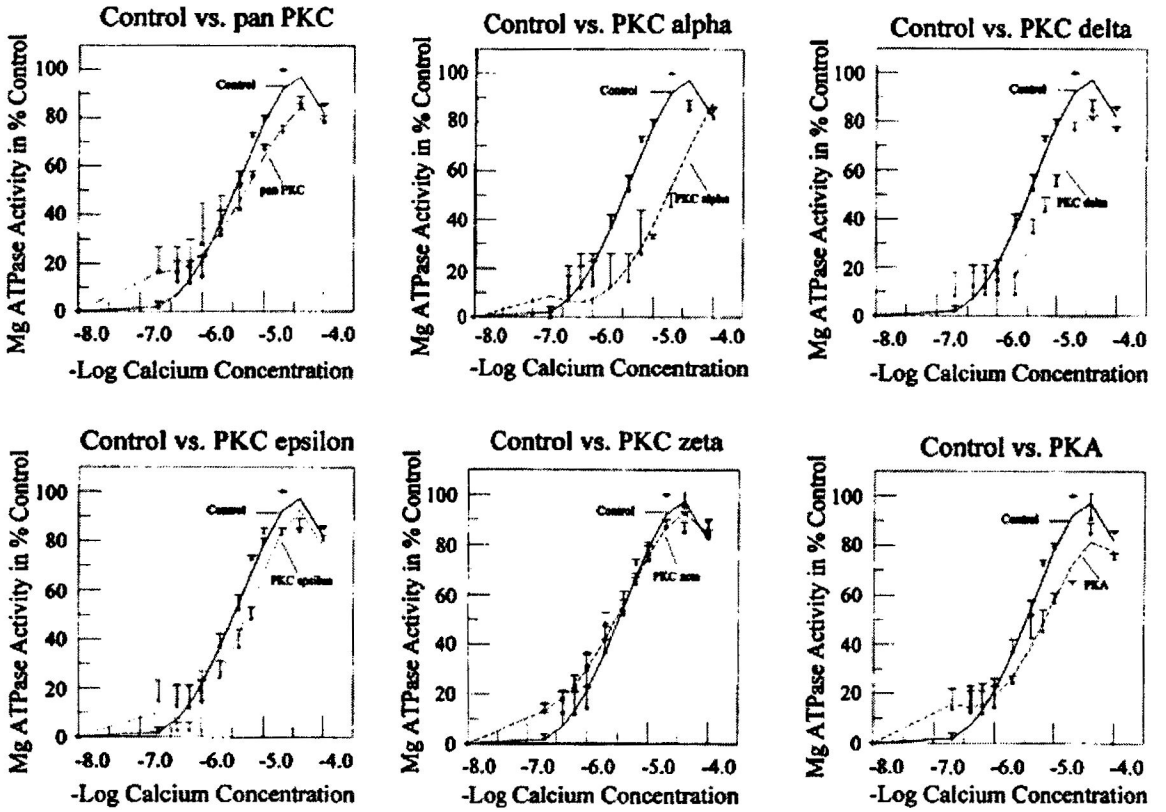

Figure 6. Effects of *in vitro* phosphorylation by PKC isozymes and PKA on Ca^{2+} sensitivity and Mg^{2+}ATPase activity in rat cardiomyofibrils. Phosphorylated and unphosphorylated myofibrils were incubated at 37°C for 10 minutes in the presence of varying Ca^{2+} concentrations (0.01-100 mM). The findings were confirmed in another set of experiments.

Table 2. Kinetic data for Ca^{2+}-stimulated Mg^{2+}ATPase activity and Ca^{2+}sensitivity of rat cardiac myofibrils phosphorylated by PKC isozymes and PKA.

Phosphorylation	Maximal Ca^{2+}-Stimulated Mg^{2+}ATPase Activity (Vmax)	EC_{50} for Ca^{2+} (Km)
Enzyme	% Control	μM
None (Control)	100	3.2 ± 0.12
pan PKC	85	3.3 ± 0.43
PKC -α	88	16.5 ± 0.35†
PKC -δ	82	5.0 ± 0.15†
PKC -ε	84	5.3 ± 0.47†
PKC -ζ	96	1.9 ± 0.13†
PKA	91	5.0 ± 0.11†

† indicates values significantly different from the control (Student's *t*-test, $p < 0.05$).

DETERMINATION OF *IN SITU* MECHANICAL CHANGES OF CARDIOMYOCYTES

Another point of interest was to observe the effect of phosphorylation on the rate of myocytic contraction. In continuation with the *in situ* studies, alterations in cardiomyocyte contraction associated with the stimulation or inhibition of the activity of PKC isozymes and PKA were evaluated by incubating isolated, viable cardiomyocytes in isoproterenol, phenylephrine, TPA, or chelerythrine chloride. The incubations were followed by observation using a trinocular microscope with a CCD camera that fed the video into a digital video software program, thus recording contractile movement (Figure 7). The mean velocities of cardiomyocytes treated with isoproterenol and phenylephrine were both higher (6.00×10^{-5} m/s and 3.47×10^{-5} m/s, respectively) than the control (untreated) cardiomyocytes, which had a mean velocity of 2.60×10^{-5} m/s (Figure 8; Table 3). Cardiomyocytes treated with chelerythrine chloride displayed a mean velocity that was higher than the control velocity, however the increase in velocity associated with this treatment was shown to be non-significant. Contrarily, cardiomyocytes treated with TPA displayed a reduction (1.67×10^{-5} m/s) in the mean velocity of contracting cardiomyocytes in comparison to the control.

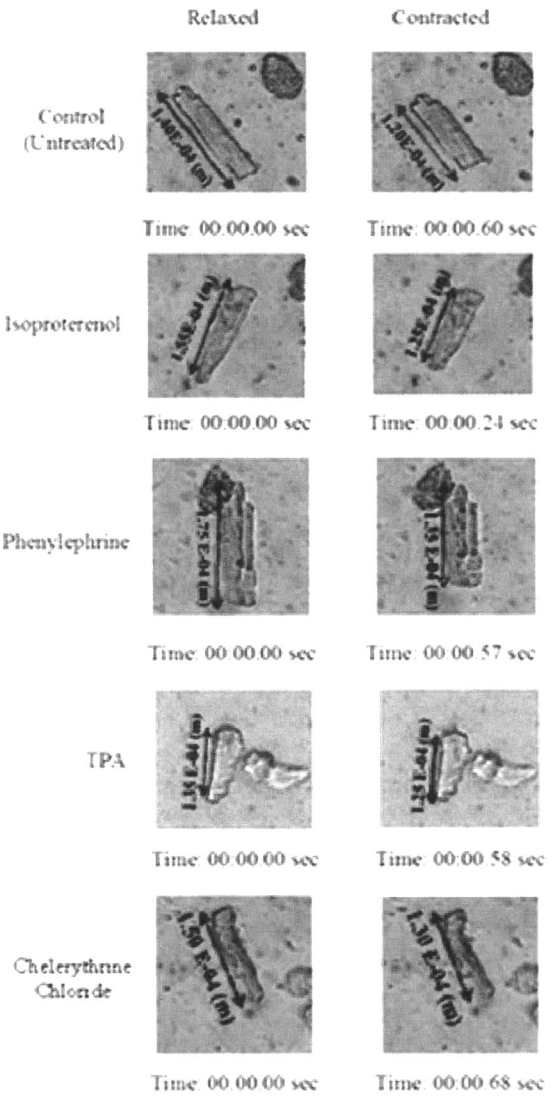

Figure 7.Visualization of relaxed and contracted cardiomyocytes treated with isoproterenol, phenylephrine, TPA, or chelerythrine chloride. Isolated myocytes were incubated in different treatments and observed using a CCD camera on a trinocular microscope. The video of contracting myocytes was replayed while the time and distances between relaxed and contracted phases were obtained.

Table 3. Velocity of contracting isolated myocytes treated with isoproterenol, phenylephrine, TPA, or chelerythrine chloride.

	Control (Untreated)	Phenylephrine	TPA	Isoproterenol	Chelerythrine Chloride
Mean Velocity (m/s)	$1.67 \times 10^{-5} \pm 0.17$	$2.17 \times 10^{-5} \pm 0.93$	$1.00 \times 10^{-5} \pm 0.29$	$1.83 \times 10^{-5} \pm 0.60$	$1.50 \times 10^{-5} \pm 0.29$
Mean Distance (m)	0.650 ± 0.05	0.713 ± 0.18	0.575 ± 0.06	0.390 ± 0.08	0.560 ± 0.15
Mean Time (s)	$2.60 \times 10^{-5} \pm 0.32$	$3.47 \times 10^{-5} \pm 0.30$	$1.67 \times 10^{-5} \pm 0.22$	$6.00 \times 10^{-5} \pm 0.87$	$2.89 \times 10^{-5} \pm 0.48$

The video of contracting cardiomyocytes, subjected to different treatments, was used to measure the relaxed length, contracted length, and contraction time. These values were used to calculate the velocity of the contracting cardiomyocytes.

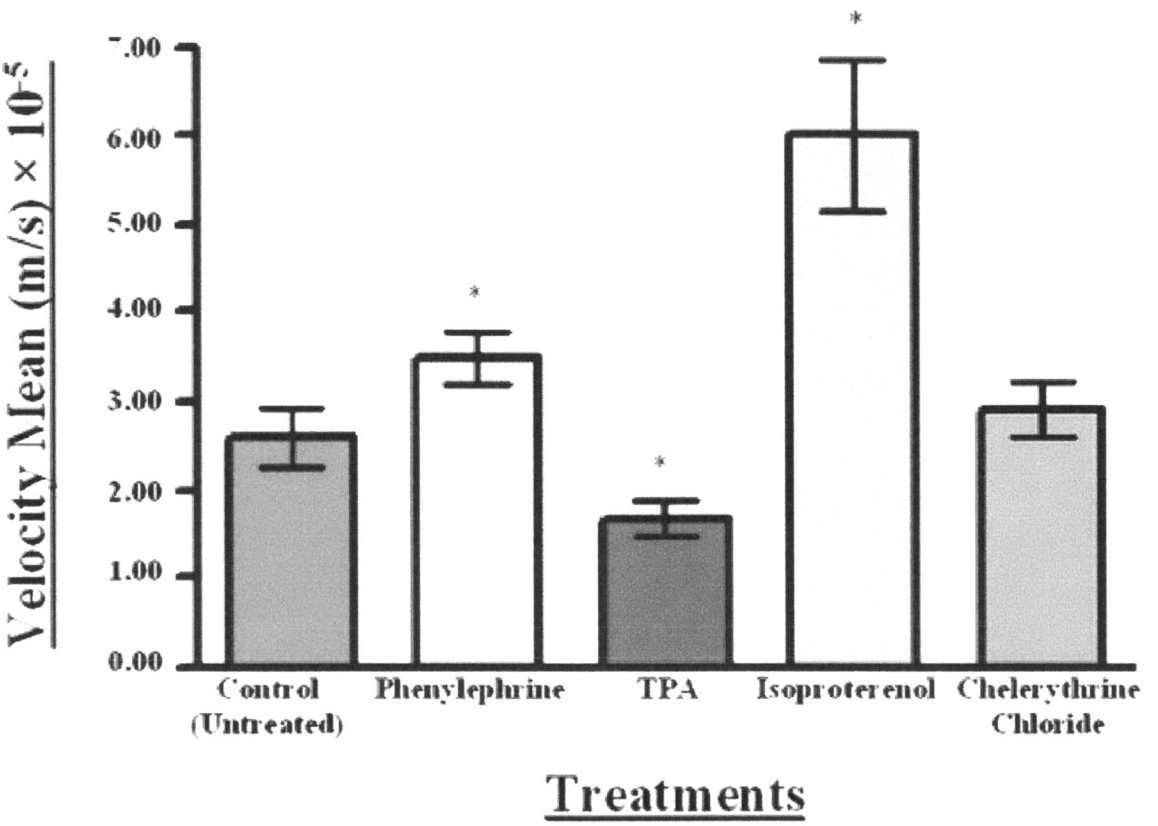

Figure 8. Velocity of contracting myocytes treated with isoproterenol, phenylephrine, TPA, or chelerythrine chloride. Isolated myocytes were incubated in different treatments and placed on slides to observe contractile movement using a video camera on a trinocular microscope. The video of contracting myocytes was played back while measurements were taken to calculate the velocity of myocytic contraction. The values were expressed as the mean of three different experiments. The * indicates values significantly different from the control.

CHAPTER 5

DISCUSSION

IN VITRO SITE SPECIFIC PHOSPHORYLATION OF CONTRACTILE PROTEINS BY PKC ISOZYMES AND PKA

This study presented evidence that PKC isozymes (α, δ, ε, and ζ) and PKA displayed distinct substrate and functional specificities in phosphorylating adult male rat ventricular myofibrils. It is important to note that in intact adult rat ventricular myofibrils (21 μg used as substrate), PKC-α phosphorylated TnT more than TnI and caused a drastic decrease in myofibrillar Ca^{2+} sensitivity. This finding revealed that phosphorylation of intact ventricular myofibrils by PKC-α may have a dual function in regulating cardiac physiology. The dual functions are prompted by (a) the decrease in myofibrillar calcium sensitivity and (b) the negative feedback inhibition caused by TnI substrate inhibition. On the other hand, PKC-ζ phosphorylated mostly TnT in the cardiac myofibrils and sharply increased myofibrillar Ca^{2+} sensitivity. Therefore, it is very important to address the physiological parameters of these isozymes in regulating cardiac function.

It has been previously reported (Noland et al. 1995, 25445-25454) that in reconstituted troponin (Tn), PKC-α phosphorylated TnI more than TnT at concentrations less than 20 μM. At concentrations above 20 μM, TnI exhibits substrate inhibition to this

isozyme. These findings confirmed the above in intact ventricular myofibrils and further delineate specific functions of these isozymes.

IN SITU SITE SPECIFIC PHOSPHORYLATION OF CONTRACTILE PROTEINS BY PKC ISOZYMES AND PKA

It has been previously reported that the role of phosphorylation in cardiac muscle physiology was to regulate Ca^{2+}-stimulated Mg^{2+}ATPase activities and calcium sensitivity (Noland et al. 1996, 14923-14931; Jideama et al. 1996, 23277-23289). The correlations between *in situ* phosphorylation of adult rat cardiomyocytes and its contractile velocity are unclear. The *in situ* phosphorylation experiments performed in this study were able to support the *in vitro* study results by displaying continuity in the site-specific phosphorylation patterns. Figure 1 showed that PKA (activated by the β-adrenergic pathway) predominately phosphorylates the TnI subunit. Figure 4 displayed that when treated with isoproterenol (β-adrenergic agonist), cardiomyocytes show increased phosphorylation on the TnI subunit. Phenylephrine (α-adrenergic agonist) showed similar supporting results by causing an increase in the phosphorylation of TnT and TnI (Figures 4, 5a, and 5b; Table 1), which have been indicated by Figure 1 to be sites for PKC isozymes (beneficiaries of the α-adrenergic pathway). The use of prazosine, an α-adrenergic antagonist, showed an expected decrease in the phosphorylation of TnT and TnI (Figures 4, 5a, and 5b; Table 1), indicating that the α- and β- adrenergic pathways play a role in the phosphorylation of contractile components in adult rat cardiomyocytes.

Treatments involving TPA, activator of diacylglycerol dependent PKC isozymes,

accordingly showed an increase in TnI and TnT phosphorylation. Chelerythrine chloride treated cardiomyocytes displayed decreased phosphorylation of TnT and TnI, which was expected due to its ability to non-selectively inhibit PKC isozymes. Gö6976 treated cardiomyocytes displayed a minor increase in TnT and TnI phosphorylation.

Although Gö6976 is an inhibitor of Ca^{2+}-dependent PKC isozymes (α, β_I, β_{II}, and γ), the remaining active PKC isozymes present in the cardiomyocytes (δ, ε, and ζ) are still capable of phosphorylating the troponin subunits. The absence of the Ca^{2+}-dependent PKC isozymes may serve to decrease the competition for the phosphorylation of the subunits, which may therefore result in the increased phosphorylation.

FUNCTIONAL SIGNIFICANCES OF CONTRACTILE PROTEIN PHOSPHORYLATION BY PKC ISOZYMES AND PKA

It is most likely that PKC-ζ phosphorylation of TnT in cardiac myofibrils occurs at the onset of muscle contraction, when the extracellular calcium is minimal. Sharp increases in calcium ion sensitivity are needed to catalyze the chemical reactions necessary to promote the sliding of actin and myosin along one another, also known as muscle contraction. These chemical reactions include: (1) phosphorylation of TnI and TnT by PKC isozymes, (2) calcium release and binding to TnC, and (3) the detachment of tropomyosin from TnT. Since PKC-ζ phosphorylation of cardiac myofibrils increased Ca^{2+} sensitivity, its function may be linked to the initiation of muscle contraction. At the resting potential of cardiac muscle, tropomyosin binds to TnT and calcium is stored in the sarcoplasmic reticulum. During action potential, Ca^{2+} is released and binds to TnC causing the detachment of tropomyosin from TnT and initiating muscle contraction. The

release of calcium from the sarcoplasmic reticulum and its binding to TnC can be associated with the increase in Ca^{2+}-sensitivity, as indicated by the differences in the Ca^{2+}-sensitivity of unphosphorylated cardiomyofibrils (3.2 μM) and the PKC-ζ phosphorylated cardiomyofibrils (1.9 μM) (Table 2). The increase in calcium sensitivity is most likely due to PKC-ζ phosphorylation of the cardiomyofibril proteins, since smaller quantities of Ca^{2+} are required for reaching maximum activity.

Data from this study also explicitly showed that by using 21 μg of cardiac myofibrils as substrate, PKC-α phosphorylated TnT more than TnI, suggesting the dual functions of PKC-α phosphorylation of the myofibrils, as mentioned above. These functions are: (a) negative feedback control of cardiac muscle contraction due to substrate inhibition of TnI and (b) a decrease in Ca^{2+} sensitivity, as indicated by the differences in unphosphorylated cardiomyofibrils (3.2 μM) and PKC-α phosphorylated cardiomyofibrils (16.5 μM) (Table 2). In the present study, the TnI substrate inhibition is likely to function as a switch mechanism from optimal cardiac muscle contraction to a relaxation phase. The observed result of PKC-α phosphorylation decreasing myofibrillar Ca^{2+} sensitivity (Table 2) suggests that functionally, PKC-α is activated during large Ca^{2+} influx into the sarcolemma. Specifically, this large Ca^{2+} influx comes from the T-tubules, which releases large quantities of Ca^{2+} directly into the extracellular fluid of the cardiac muscle. These calcium ions are released during the action potential and are needed for maximal contraction. This suggests that TnI substrate inhibition occurs at the end of cardiac muscle contraction, when the influx of calcium ions into the muscle fiber is

maximal. In order to avoid hyper-contractility, calcium is pumped back into the sarcoplasmic reticulum and T-tubules, therefore, stopping contraction. The ability of PKC-α phosphorylation to decrease calcium sensitivity and to cause TnI substrate inhibition suggests that this isozyme is a calcium regulator in cardiac muscle contractility. It also suggests that it initiates the inhibitory effect of TnI, and functions to terminate each cardiac muscle contractile phase by causing substrate inhibition of the inhibitory subunit, TnI.

While PKC-α phosphorylation proposes to function as a terminator of each cardiac contractile phase, PKC-ζ phosphorylation of ventricular myofibrils is likely to initiate cardiac muscle contraction. Since PKC-ζ phosphorylated mostly TnT, the binding site of tropomyosin during the resting potential (non-contracting phase), it is suggested that its mechanism of action results in the conformational change, which is thought to tug on the tropomyosin molecule leading to its release from TnT of the actin filament. Also, phosphorylation of TnT by PKC-ζ increased calcium sensitivity, making calcium available for binding at TnC. This calcium release and binding to TnC is believed to initiate cardiac muscle contraction, implicating PKC-ζ to initiation of cardiac muscle contraction. Contrarily, PKC-α, which phosphorylated TnI more than TnT at low concentrations and exhibits substrate inhibition of TnI at high concentrations, reduced calcium sensitivity probably when TnC, the calcium-binding site, is fully saturated. This implies that PKC-α phosphorylation inhibits further release of calcium from sarcoplasmic reticulum and T-tubules and its binding to TnC, therefore, terminating cardiac muscle contraction. It is most likely that substrate inhibition of TnI, which occurs

as a result of PKC-α phosphorylation, occurs concurrently with the decrease in calcium level. Substrate inhibition of TnI (the binding site for actin) phosphorylation by PKC-α may be the mechanism necessary for the inhibition of the actin filament by troponin-tropomyosin complex. In relaxed cardiac muscle, the active sites of actin filament are physically covered by the troponin-tropomyosin complex, which prevents the attachment of the heads of the myosin filaments. Therefore, PKC-α phosphorylation of TnI at low concentrations, when TnI is phosphorylated more than TnT, and PKC-ζ phosphorylation of TnT, which increases calcium sensitivity, may be implicated to the physiological mechanism needed for the removal of troponin-tropomyosin complex from the active sites of actin filament. On the other hand, decrease in calcium sensitivity and substrate inhibition of TnI phosphorylation by PKC-α may be essential parameters for troponin-tropomyosin complex binding to the active sites of the actin filament causing the muscle to relax.

MECHANICAL EFFECTS ON CARDIOMYOCYTE PHYSIOLOGY ASSOCIATED WITH PKC ISOZYMES AND PKA

This study also determined the effects of inducing the activation of PKC and PKA on the cardiomyocytic velocities. The correlation between *in situ* phosphorylation and contractile velocities of adult rat cardiomyocytes treated with α- and β- adrenergic signaling pathway agonists (phenylephrine and isoproterenol, respectively) showed some significant differences in the contractile velocities of the cardiomyocytes. Stimulation of each pathway increased the contractile velocity over 25%, when compared to the control (untreated) myocytes. This suggests that though the pathways are specific in the

activation of these enzymes, they both have similar effects on the mechanical properties of the contractile proteins.

As previously mentioned, the *in vitro* studies revealed that PKA shows substrate specificity in phosphorylating the troponin Mg^{2+} ATPase inhibitory subunit (TnI), while the PKC isozymes phosphorylate TnT preferably higher than TnI. Treatment with the phorbol ester (TPA) resulted in a decrease in contractile velocity. TPA is commonly used as an activator of PKC. TPA is a non-hydrolysable, non-physiological analogue structurally similar to diacylglycerol that, unlike diacylglycerol, it is not readily degraded and continues to activate diacylglycerol dependent isozymes of PKC (α, β_I, β_{II}, δ, and ε). The decrease in contractile velocity after TPA treatment (Figure 8; Table 3) may be a result of increased phosphorylation of the TnT and the decreased phosphorylation in the TnI component of the troponin complex. Furthermore, chelerythrine chloride was used to evaluate the effect of PKC inhibition. It was observed that chelerythrine chloride treated cardiomyocytes (which caused a decrease in TnT phosphorylation and an increase in TnI phosphorylation) displayed a velocity almost twice that of TPA treated cardiomyocytes. This suggests that the PKC isozymes may display a regulatory role in keeping myocytes contraction from reaching high velocities that can over work the heart, leading to cardiac arrest. The β-adrenergic (PKA activation pathway) influenced *in situ* phosphorylation of TnI was much more than that of the control, phenylephrine, and TPA treated cardiomyocytes, while its phosphorylation of TnT was close to being significantly less than the control. Interestingly, the contractile velocities of these β-adrenergic influenced *in situ* phosphorylated cardiomyocytes were the highest among the other treated

cardiomyocytes.

The lack of significant differences in the *in situ* phosphorylation of TnT (Figures 5a and 5b) suggests that the *in situ* phosphorylation of the TnI component has a distinguished effect on increasing the contractile velocity of the cardiomyocytes. This data cooperatively suggests that the contractile velocity of cardiomyocytes increases with the phosphorylation intensity of TnI and decreases with the phosphorylation intensity of TnT.

SUMMARY AND FUTURE STUDIES

The data from this study has collectively revealed that the phosphorylation of adult rat cardiomyocytes by various PKC isozymes and PKA have (a) specific substrates on cardiomyofibrils and (b) display alternating effects on the physiological aspects of cardiomyocyte function. It is anticipated that future experiments will make use of this data by attempting to manipulate the contraction of cardiomyocytes in *in vivo* experiments. These experiments would potentially involve the use of hypertensive rats, whereby the goal would be to alter the contractile patterns of the heart by manipulating the involvement of the adrenergic pathways.

Previously, it has been reported that PKA phosphorylates TnI at serine 23/24 (Noland et al. 1996, 14923-14931). It therefore becomes apparent from this data that the contractile velocity of the cardiac muscle may be optimum with the amino acid terminal end phosphorylation of TnI at the serine 23/24 phosphorylation site, which is characterized by PKA. Phosphorylation alone is not as important as the type and location of phosphorylation. PKC and PKA phosphorylate at different sites within a protein

sequence. Further studies may reveal the precise function of these phosphorylation sites by deletion or addition of amino acids within these sites, which may lead to a better understanding of the roles each kinase has in cardiomyopathies.

Future studies will also involve the evaluation of an additional enzyme, myosin light chain kinase (MLCK), and its substrate, myosin light chain 2 (MLC2). In addition to TnI and TnT in the thin filament, phosphorylation of MLC2 in the thick filament is also involved in the regulation of muscle contraction. Abnormal cardiac structure and function in mice has been linked to non-phosphorylatable regulatory MLC2 (Sanbe et al. 1999, 21085-21094). The 20 kDa MLC2 in vertebrate non-muscle and muscle cells is phosphorylated by a specific Ca^{2+}/calmodulin-dependent MLCK (Solero 2000, 173; Sellers et al. 1987, 381-418). MLCK mediated phosphorylation in smooth muscle is associated with a greater than 100-fold (Sellers 1985, 15815-15819) increase in actin-activated Mg^{2+}ATPase activity of myosin and is a primary mechanism for initiation of contraction. Furthermore, MLC2 is also a substrate for PKC. PKC incorporates phosphate into smooth muscle or non-muscle MLC2 *in vitro* and *in situ* at sites that are distinct from the MLCK sites (Jideama et al. 2006, 1-9; Ludowyke et al. 1989, 12492-12501). PKC-mediated phosphorylation has also been shown to inhibit increases in Mg^{2+}ATPase activity associated with phosphorylation by MLCK (Bogatcheva et al. 2003, L415 - L426; Nishikawa et al. 1984, 8808-8814). The physiological significance of this phosphorylation has not been clearly defined. Therefore, it is hoped that these future studies may be used to bring more understanding to the collective role of kinases and their substrates in the regulation of contractile function.

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